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Molecular cloning and expression analysis of interferon regulatory factor-1 (IRF-1) of turbot and sea bream

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Abstract

The interferon regulatory factor (IRF) family comprises transcription factors that regulate the expression of interferon and interferon-related cytokines. Using the RACE technique, we have determined the complete cDNA sequence of turbot (*Scophthalmus maximus*) and sea bream (*Sparus aurata*) IRFs. These sequences shared characteristics with other IRFs of fish, mammals and birds, and showed high similarity with IRF-1. Indeed, they were included in the IRF-1 cluster of the phylogenetic tree constructed with IRF-1 and IRF-2 sequences of several organisms, and presented a low number of basic amino acid residues in the carboxy-terminal end of the proteins. All of these characteristics led to the identification of turbot and sea bream IRFs as IRF-1. Two IRF-1 sequences were obtained for both turbot and sea bream, and we named them turbot/sea bream IRF-1a and IRF-1b. Turbot IRF-1a differed from turbot IRF-1b in four nucleotides. The presence of both IRF types in cDNA from 45 turbot livers was determined by RFLP, suggesting the duplication of the gene. Sea bream IRF-1b presented a deletion of 121 bp in its ORF compared to sea bream IRF-1a, and since both IRF types were present in all 25 cDNAs analyzed by PCR, we hypothesized that the truncated sea bream IRF-1b was probably an alternative splicing product. Turbot and sea bream IRF-1 expression was constitutive in every analyzed organ, as reported before for other fish species. Poly I:C significantly stimulated turbot IRF-1 expression in muscle, spleen and kidney 24 h post-treatment, while viral haemorrhagic septicemia virus (VHSV) induced a differential expression of this factor in kidney 8 h after infection. These results do not agree with those previously reported for flounder and trout IRF. Other expression experiments with turbot leukocytes stimulated in vitro with poly I:C and with brain and kidney of sea bream infected with nodavirus did not bring out differential IRF expression levels in stimulated samples with respect to controls.

Keywords: IRF-1; Interferon regulatory factor; Cytokine; Teleostei; Turbot; Sea bream; VHSV

1. Introduction

Type I interferons (IFNs) are a family of 165–172 amino acid proteins secreted by leukocytes and fibroblasts, that can modulate several cellular functions including cell growth and differentiation, immune system and viral replication (Donnelly, 1994). The antiviral activity of IFNs, characteristic that led to their discovery (Isaacs and Lindenmann, 1987), is mediated by the action of interferon-induced proteins, such as Mx proteins or major histocompatibility (MHC) antigens (reviewed in Kroger et al., 2002; Samuel, 1991). The activity of IFN and IFN-inducible proteins is modulated by IFN regulatory factors (IRFs), family of transcription factors that can be classified in four groups: activators (IRF-1 and ISGF3), repressors (IRF-2, ICSBP), activators and repressors (IRF-4), and activators or repressors (IRF-3) of target genes transcription (Nguyen et al., 1997). The typical IRF recognition sequences, usually known as ISREs (interferon stimulating response elements), are highly conserved, and their motifs are well known. These ISREs are located in the promoters of many IFN- and virus-inducible genes (Tanaka et al., 1993). Regarding teleost fish, it has been recently identified an ISRE in the rainbow trout (*Oncorhynchus mykiss*) Mx1 promoter (Collet and Secombes, 2001).

The most studied IRFs are IRF-1 and IRF-2, structurally similar proteins with high sequence homology in their N-terminal region, where resides the DNA binding domain

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(Harada et al., 1989). In contrast, the C-terminal region of IRF-1, where the transcriptional activation domain is located (Fujita et al., 1989), shows very low homology with that of IRF-2. This suggests the possible function of IRF-2 as a controlling factor for IRF-1 by binding site competition (Nguyen et al., 1997), which explains the reported activity of IRF-1 as a transcriptional activator, and IRF-2 as a suppressor of the IRF-1 function (Harada et al., 1989). Interestingly, although IRF-2 is generally considered as a transcriptional repressor, there are some studies in which it acts as an activator, so it has been proposed to be a dual transcription factor containing both activator and repressor functions (Nguyen et al., 1997). Among the physiological roles of IRF-1 and IRF-2, the antiviral defense, immune regulation and cell growth control should be highlighted (Nguyen et al., 1997).

Little is known on the nucleotide sequence and activity of the interferon gene in teleosts. In fact, this molecule has only been characterized in pufferfish (*Tetraodon nigroviridis*) (Lutfalla et al., 2003), zebrafish (*Danio rerio*) (Altmann et al., 2003), Atlantic salmon (*Salmo salar*) (Robertsen et al., 2003), and channel catfish (*Ictalurus punctatus*) (Long et al., 2004), while its antiviral activity has only been demonstrated for the last three fish species. There is a similar lack of knowledge in teleosts with respect to IRF: it has been characterized to date in Japanese flounder (*Paralichthys olivaceus*) (Yabu et al., 1998), pufferfish (*Fugu rubripes*) (Richardson et al., 2001), and, very recently, in rainbow trout (*O. mykiss*) (Collet et al., 2003). In this paper, we clone, sequence and analyze the expression of IRF in turbot (*Scophthalmus maximus*) and sea bream (*Sparus aurata*), two marine fish species of growing commercial interest in Spain.

2. Materials and methods

2.1. Experimental animals

Turbot (*S. maximus*) of 500 g and sea bream (*S. aurata*) of 100 g mean total weight, were obtained from a commercial fish farm in Galicia (NW Spain). The animals were acclimatized to laboratory conditions for 1 month before being used for the experiments. They were maintained at 18 °C and fed daily with a commercial diet.

2.2. RNA extraction and generation of full-length cDNA

Turbot and sea bream were intraperitoneally (i.p.) injected with poly (I)–poly (C) (poly I:C) (Sigma) (250 μl of 5 mg ml^{-1} per animal), a sea bass nodavirus strain (only for sea bream, 200 μl of 1×10^6 $TCID_{50}$ ml^{-1} per animal), or phosphate buffered saline (PBS). Twenty-four hours after injection, fish were anaesthetized and organs were extracted for RNA isolation with Trizol (Life Technologies). Five micrograms of RNA were used to obtain cDNA using the SuperScript II RNAase H-Reverse Transcriptase (Invitrogen), and the resulting cDNA was stored at −20 °C.

The pair of primers TRU IRF 1-S (5′-TGAGAATGAGACCTTGGTTGG-3′)/TRU IRF 1-AS (5′-TTGAACAGACATGCGTCCTT-3′), derived from conserved regions of the trout IRF-1 gene (GenBank accession number AF332147), was used for amplification of turbot and sea bream IRF by PCR of the previously obtained cDNAs. PCR products that produced a band of the expected size (150 bp) were cloned using pGem-T Vector System (Promega) and Top 10F′ competent bacteria (Invitrogen), and several clones were sequenced from both ends with M13 forward and reverse primers using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in an ABI Prism 377 automatic sequencer (Perkin-Elmer). Sequences with strong homology with other known IRF sequences were obtained from spleen cDNA of one control turbot and from kidney cDNA of one nodavirus infected sea bream, and these sequences were used to design primers for 5′- and 3′-RACE cDNA synthesis. RACE reactions were performed using SMART RACE cDNA Amplification Kit from Clontech according to the manufacturer's instructions. In order to check the full length IRF sequences obtained by RACE, primer pairs IRF ROD DEF-F (5′-TGTTCACTCTTGAGCAGCAAA-3′)/IRF ROD DEF-R (5′-TGTATAAAAACACACAAACAATAACAA-3′) and IRF DORADA DEF-F (5′-GGACAGTACCCGCTTCTGAT-3′)/IRF DORADA DEF-R (5′-CAAACACACAAACAAAACAATCTG-3′) were designed to perform a RT-PCR with the cDNAs previously obtained, and several clones were completely sequenced at least twice for each fish species. All primers used for PCR and DNA sequencing were designed with the program Primer3 (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi) (Rozen and Skaletsky, 2000).

2.3. Expression analysis of turbot and sea bream IRF

To study the turbot IRF expression in vivo, animals were first i.p. inoculated with 250 μl of 5 mg ml^{-1} poly I:C (or PBS for the controls) and, 24 h afterwards, muscle, liver, spleen, kidney, gills and blood leukocytes (Graham and Secombes, 1998) were extracted for RNA isolation (Trizol). In another experiment, turbot were i.p. inoculated with 100 μl of viral haemorrhagic septicemia virus (VHSV) (10^7 $TCID_{50}$ ml^{-1}), bacteria *Vibrio pelagius* strain Hq222 (Villamil et al., 2003) (10^6 CFU ml^{-1}) or PBS for the controls. Four animals from each treatment were sampled 8, 24 and 48 h post-inoculation, and kidney and liver were extracted for RNA isolation with Trizol.

To study the turbot IRF expression in vitro, leukocytes from healthy turbot were isolated (Graham and Secombes, 1998), treated with 50 μg ml^{-1} poly I:C (or left without treatment for the controls) for different times at 20 °C, and taken for RNA extraction with Trizol.

On the other hand, sea bream IRF expression was studied in kidney and brain of animals intramuscularly and intracerebrally infected with a nodavirus strain 1, 3 and 7 days before organs extraction for RNA isolation with Trizol.